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Three Pathogenicity Islands of *Vibrio cholerae* Can Excise from the Chromosome and Form Circular Intermediates▽†

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***Vibrio* pathogenicity island-2 (VPI-2) is a 57-kb region integrated at a transfer RNA (tRNA)-serine locus that encompasses VC1758 to VC1809 on the *V. cholerae* N16961 genome and is present in pandemic isolates. VPI-2 encodes a P4-like integrase, a restriction modification system, a Mu phage-like region, and a sialic acid metabolism region, as well as neuraminidase (VC1784), which is a glycosylhydrolase known to release sialic acid from sialoglycoconjugates to unmask GM1 gangliosides, the receptor for cholera toxin. We examined the tRNA-serine locus among the sequenced *V. cholerae* genomes and identified five variant VPI-2 regions, four of which retained the sialometabolism region. Three variant VPI-2 regions contained a type three secretion system. By using an inverse nested PCR approach, we found that the VPI-2 region can form an extrachromosomal circular intermediate (CI) molecule after precise excision from its tRNA-serine attachment site. We constructed a knockout mutant of VC1758 (*int*) with *V. cholerae* strain N16961 and found that no excision PCR product was produced, indicating that a functional cognate, VPI-2 integrase, is required for excision. The *Vibrio* seventh pandemic island-I (VSP-I) and VSP-II regions are present in *V. cholerae* O1 El Tor and O139 serogroup isolates. Novel regions are present at the VSP-I insertion site in strain MZO-3 and at the VSP-II insertion site in strain 623-39. VSP-II is a 27-kb region that integrates at a tRNA-methionine locus, is flanked by direct repeats, and encodes a P4-like integrase. We show that VSP-II can excise and form a CI and that the cognate VSP-II integrase is required for excision. Interestingly, VSP-I is not inserted at a tRNA locus and does encode a XerDC-like recombinase, but similar to VPI-2 and VSP-II, VSP-I does excise from the genome to form a CI. These results show that all three pathogenicity islands can excise from the chromosome, which is likely a first step in their horizontal transfer.**

Horizontal gene transfer (HGT) and the acquisition of foreign DNA is a fundamental process in the evolution of most bacterial species. The acquisition of mobile genetic elements such as plasmids, bacteriophages, transposons, integrative and conjugative elements, and genomic islands (GEIs) allows bacteria to instantly obtain a range of genetic traits that may increase fitness under different environmental conditions. GEIs are classified based on the different functions they encode, which include metabolic islands, degradation islands, resistance islands, symbiosis islands, and pathogenicity islands (PAI) (7–9). The sequences of GEIs display certain properties that mark them as being atypical compared to the overall genome of the organism in which they are found. These features include (i) a large chromosomal region present in a subset of isolates of a species and absent from closely related isolates, (ii) the presence of mobility genes such as integrases and transposases, (iii) association with a tRNA gene, (iv) flanking direct repeat sites, which mark the sequence where the incoming DNA recombined with the genome, (v) a G+C content which differs significantly from that of the overall G+C content of the host organism, and (vi) instability in their chromosomal insertion sites (7–9). The mechanism(s) of acquisition used by these GEIs is still poorly understood, although one of three primary mechanisms, conjugation, transduction, and transformation, is involved.

V. cholerae O1 and O139 serogroup isolates harbor four PAIs: *Vibrio* pathogenicity island-1 (VPI-1), VPI-2, *Vibrio* seventh pandemic island-I (VSP-I), and VSP-II (4, 14, 16, 19, 25). VPI-1 is a 41-kb region that integrates at the tmRNA (*ssrA*) loci, a common site for the integration of PAIs in *Vibrio* species (16, 19, 31). VPI-1 encodes the toxin-coregulated pilus (TCP), an essential colonization factor, the accessory colonization factor (ACF), and the virulence regulators ToxT and TcpPH (16, 19). Subsequently, it was proposed that VPI-1 encoded a novel filamentous phage required for its mobility and interstrain transfer (17). However, ensuing research found that this was not the case (5, 30). O'Shea and Boyd demonstrated that VPI-1 can be transferred via generalized transduction between *V. cholerae* serogroup O1 strains (24). VPI-1 has the ability to excise from its chromosomal insertion site and circularize to form a circular intermediate (CI); however, the cognate integrase was not essential for excision (30).

VPI-2 is a 57.3-kb PAI consisting of 52 open reading frames (ORFs), VC1758 to VC1809, on the *V. cholerae* N16961 genome (14). Jermyn and Boyd showed that VPI-2 displays all the characteristics of a horizontally transferred PAI: a G+C content which diverges from that of the whole genome (42% for VPI-2 versus 47% for the *V. cholerae* genome), the presence of a P4-like integrase (VC1758), and a chromosomal insertion at a tRNA-serine (VC1757.1) locus that is flanked by direct repeats, and it is present in pathogenic strains of *V.*

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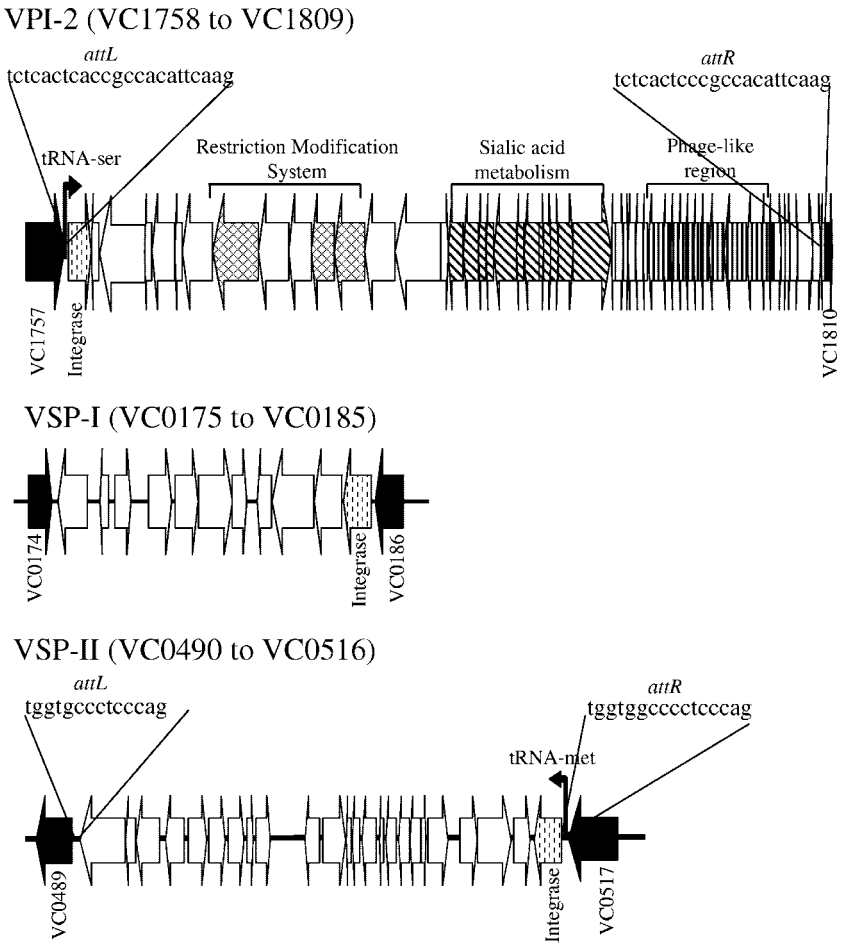

FIG. 1. Schematic representation of VPI-2, VSP-I, and VSP-II from *V. cholerae* strain N16961 (10). Black arrows represent core chromosomal genes, and open arrows represent island region genes. Dashed arrows indicate integrase genes, cross-hatched arrows indicate type I restriction modification genes, diagonally striped arrows indicate sialic acid metabolism genes, vertically striped arrows represent Mu phage genes, and vertical arrows represent tRNA loci. The *attL* and *attR* genes indicate left and right attachment sites.

cholerae (Fig. 1) (14, 15). VPI-2 encodes a type 1 restriction modification (RM) system and a region that shows homology to a Mu phage (14, 15). VPI-2 also encodes sialic acid transport (ORFs VC1777 to VC1779) and catabolism homologues (ORF VC1776 and VC1781 to VC1783), as well as neuraminidase (ORF VC1784), which converts higher-order sialogangliosides to GM1 gangliosides, the receptor for cholera toxin, with the release of sialic acid (6). *V. cholerae* neuraminidase may also form part of the mucinase complex that hydrolyzes intestinal mucus, enabling the bacterium to move readily to the epithelium (32). Interestingly, none of the four additional published *Vibrio* species genomes encodes neuraminidase, but they do contain the genes for de novo synthesis of sialic acid, which are absent from *V. cholerae* (31). VSP-I is a 16-kb region spanning ORFs VC0175 to VC0185 (4). The VSP-I region has an atypical G+C content of 40% (versus 47% for the entire genome) and is present only in the O1 El Tor and O139 serogroup isolates (4). Of the 11 genes carried by VSP-I, 7 genes encode hypothetical proteins, and VC0185 encodes a putative XerDC-like integrase (4).

VSP-II is a 27-kb region integrated at a tRNA-methionine locus (VC0516.1) and encodes a P4-like integrase (25). Originally, VSP-II was identified as a 7.5-kb region encompassing ORFs VC0490 to VC0497 and was found exclusively in the *V. cholerae* El Tor O1 and O139 serogroup isolates (4). Subsequently, O'Shea et al. showed that VSP-II is a much larger region, encompassing ORFs VC0490 to VC0516 (Fig. 1) (25). VSP-II encodes homologues of an RNase H1 protein, a type IV pilus, a DNA repair protein, two transcriptional regulators, two methyl-accepting chemotaxis proteins, and a P4-like integrase (VC0516) adjacent to the tRNA-methionine locus (VC0517.1) (25).

In this study, we examined the genomic structures of VPI-2, VSP-I, and VSP-II, among the sequenced *V. cholerae* genomes in the database. We uncovered considerable variability in gene content within VPI-2 and the presence of novel regions at the VSP-I and VSP-II insertion sites in strains MZO-3 and 623-39. Most of the variant VPI-2 regions identified retained the sialic acid metabolism region (VC1773 to VC1784), the P4-like-integrase region (VC1758), and the region VC1804 to VC1809 and were flanked by direct repeats. We investigated the abilities of VPI-2, VSP-I, and VSP-II to excise from their integration sites and form extrachromosomal CIs, a first step in their possible horizontal transfer. We demonstrate that all three

TABLE 1. *E. coli* strains and plasmids used in this study

Strain plasmid	Description and features	Reference or source
Vibrio cholerae strains		
O395	O1 classical, VPI-2$^+$, VSP-I and VSP-II deficient	Laboratory collection
2740-80	O1 El Tor, VPI-2$^+$, VSP-I and VSP-II deficient	Laboratory collection
N16961	O1 El Tor, VPI-2$^+$, VSP-I$^+$, VSP-II$^+$	Laboratory collection
E4	O1 El Tor, E7946 derivative	Laboratory collection
SG6	Non-O1, VPI-2 deficient, VSP-I deficient, VSP-II deficient	Laboratory collection
MO2	O139, VPI-2$^+$, VSP-I$^+$, VSP-II$^+$	Laboratory collection
MO10	O139, VPI-2$^+$, VSP-I$^+$, VSP-II$^+$	Laboratory collection
Bah-3	E7946 Δ*recA*	Laboratory collection
RAM-1	N16961 $\Delta int_{VPI\ 2}$	This study
RAM-2	N16961 $\Delta int_{VSP\ I}$	This study
RAM-3	N16961 $\Delta int_{VSP\ II}$	This study
Escherichia coli strains		
DH5αλpir		
β2155	ΔDAP *pir*	
RM1	DH5αλpir(pRM1)	This study
RM2	DH5αλpir(pRM2)	This study
RM3	DH5αλpir(pRM3)	This study
RM4	β2155(pRM1)	This study
RM5	β2155(pRM2)	This study
RM6	β2155(pRM3)	This study
RM7	DH5αλpi(pRM4)	This study
RM8	DH5αλpi(pRM5)	This study
RM10	β2155(pRM4)	This study
RM11	β2155(pRM5)	This study
Plasmids		
pDS132	*cat sacB* Δ*pir*	27
pRM1	pDS132 $int_{VPI\ 2}$ SOE construct	This study
pRM2	pDS132 $int_{VSP\ I}$ SOE construct	This study
pRM3	pDS132 $int_{VSP\ II}$ SOE construct	This study

PAIs can excise and form CIs and that excision is mediated by $int_{VPI\text{-}2}$, $int_{VSP\text{-}I}$, and $int_{VSP\text{-}II}$, encoded in VPI-2, VSP-I, and VSP-II, respectively.

MATERIALS AND METHODS

Comparative genomic analysis. The complete sequenced genome of *V. cholerae* N16961 and the genome sequences of *V. cholerae* strains O395, MAK757, B33, MZO-3, MO10, AM-19226, 1587, 623-39, NRT36S, V51, RC385, and V52 were accessed through the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/entrez) (Table 1). Homology searches were carried out using the Basic Local Alignment Search Tool (BLAST) service provided by NCBI (www.ncbi.nlm.nih.gov/BLAST). DNA sequence comparisons were performed using ClustalW alignment software provided by the European Biotechnology Institute (EBI) (www.ebi.ac.uk/clustalw). The genomes and the partial genomes of *V. cholerae* strains were compared using an online version of Artemis comparison tool (WebACT) software (www.webact.org) provided by the Sanger Institute. Genome comparisons were viewed from the database of precomputed genome comparisons (www.webact.org/WebACT/prebuilt), or sequence comparisons were generated by uploading DNA sequences directly (www.webact.org/WebACT/generate).

Bacterial strains. Seven *V. cholerae* isolates were used in this study, the O1 serogroup strains O395 (classical biotype), 2740-80 (El Tor biotype), N16961 (El Tor biotype), E4 (El Tor biotype), Bah-3 (El Tor RecA$^-$), and SG6 (non-O1) and the O139 serogroup strains MO2 and MO10 (Table 1). The two O139 isolates included strain MO2, which contains the complete VPI-2 region, and MO10, which contains a truncated version of the island (14, 15). *Escherichia coli* strains DH5αλpir and β2155 were employed in the construction of *V. cholerae* knockout mutants (Table 1). All isolates were grown in Luria-Bertani (LB) broth at 37°C. All strains were stored at −70°C in broth containing 20% (vol/vol) glycerol.

Molecular techniques. Chromosomal DNA was isolated from all *V. cholerae* strains using a G-nome DNA isolation kit (Bio 101; Q-Biogene). Primers were designed using the published genome sequence of *V. cholerae* N16961 as the template (10) (Table 2). Flanking primers were designed using the 5′ and 3′ chromosomal genes immediately before and after the first and last genes in VPI-2, VSP-I, and VSP-II. These flanking primers allowed amplification across each island region to examine the insertion sites among isolates; thus, PCR amplification occurred only if the flanking genes were contiguous and the island regions were missing and no novel region was present at these sites.

PCRs were performed in a 25- or 50-μl volume, using standard conditions. PCR products were purified using a Jetquick PCR purification system (GenoMed) or a Gel Extraction kit (Qiagen). Nucleotide sequence data were generated from PCR products, using primers described in Table 2. For example, the *attP* sequence of VPI-2 was generated by sequencing the PCR product of the NestVC1809F/NestVC1758R primer pair. Nucleotide sequencing was carried out by using MWG-Biotech (Germany) sequencing.

Isolation of VPI-2, VSP-I, and VSP-II CIs. VPI-2 and VSP-II each encode a P4-like integrase, they are inserted adjacent to the tRNA genes, and they are both flanked by direct repeats (Fig. 1). VSP-I encodes a XerDC-like integrase gene. To determine whether these PAIs are stable at these insertion sites or whether they can excise from the genome and form a CI, we performed inverse and nested PCR assays on a number of VPI-2 and VSP-I-positive and VSP-II-positive *V. cholerae* strains using primer pairs described in Table 2. A Qiagen plasmid mini-kit was used to recover plasmid DNA from the *V. cholerae* strains O395, 2740-80, N16961, E4, MO2, MO10, SG6, and Bah-3, following the manufacturer's instructions. Plasmid DNA was resuspended in 100 μl of Tris-EDTA buffer (pH 8) and stored at −20°C.

Inverse PCR. Inverse primer pairs were designed to detect the CIs of VPI-2, VSP-I, and VSP-II (Table 2). Primer InvVC1809F was designed to amplify the VC1809 gene, the last 3′ region gene of VPI-2, and primer InvVC1758R was designed to amplify the VC1758 gene, the first gene at the 5′ end of VPI-2 (Table 2; Fig. 2). This primer pair will amplify a 2,053-bp PCR product if a CI is formed after the excision of VPI-2. Similarly, primer InvVC0185F was designed to amplify the VC0185 gene, the last VSP-I gene at the 3′ end of the island, and primer InvVC0175R was designed to amplify the VC0175 gene, the first gene at the 5′ end of VSP-I (Table 2). This primer pair will amplify a 1,889-bp PCR product after excision and circularization of VSP-I. Similarly for VSP-II, primer

TABLE 2. Primers used in this study

Name	Region	Sequence (5′–3′)	Product size (bp)	Temp (°C)
Inverse				
InvVC1809F	VPI-2	ACGATTGGTGATCCGTTGAGC	2,053	60
InvVC1758R		AGAGTGGTACTTGCCAAAGC		
InvVC0185F	VSP-I	AGTAGCCCTTAAATCGTGAGTCC	1,889	57
InvVC0175R		GCGTCCATACTGGCAGATGCTCG		
InvVC0516F	VSP-II	AGCGCCATATTCGCACTCTG	1,922	59
InvVC0490R		GACACTGAAAACCCCAATGCTCC		
Nested				
NestVC1809F	VPI-2	TCACACCTCTCAGGTTGG	1,303	55
NestVC1758R		GCCGTAATGCAGCGATGG		
NestVC0185F	VSP-I	TCTCTTAAGGGCGCGAACAC	679	58
NestVC0175R		CGAAGGTGTGTCATCTCGACG		
NestVC0516F	VSP-II	AGTCGATATCCTGCCAACGTGC	1,493	59
NestVC0490R		ACACCAAGCGACTCCTGAGG		
SOE constructs				
SOEVC1758a	VPI-2	GCTCTAGAGCACTTTTGCGTACGTCCAAGC	387	60
SOEVC1758b		GCCGTAATGCAGCGATGG		
SOEVC1758c	VPI-2	CCATCGCTGCATTACGGCGCCTAACGAAGCCTCTGGTG	422	60
SOEVC1758d		GCGAGCTCGCACCAGCTCATAAGCTTTCGACG		
SOEVC0185a	VSP-I	GCTCTAGAGCAGCTAAAGCATCACCAACAGG	480	56
SOEVC0185b		ACAGTCTGGATTGCGCGTTGA		
SOEVC0185c	VSP-I	TCAACGCGCAATCCAGACTGTAAATCTCTCATGAGCTGCCCA	383	57
SOEVC0185d		GCGAGCTCGCCGAACAATTTTGACGTTCCCCGA		
SOEVC0516a	VSP-II	GCTCTAGAGCCATGAGTTTTCTGCGTTGTTCG	410	57
SOEVC0516b		TCGAGAGTGCATCAGCACGT		
SOEVC0516c	VSP-II	ACGTGCTGTAGCACTCTCGAGGTTTGATGTCTTGAATGGGGAC	386	58
SOEVC0516d		GCGAGCTCGCACTCGCTCTGATGGTGATGGCCT		
Flanking mutant genes				
fkVC1757F	VPI-2	AGAGTATGGCGGTCACCA	2,098	56
fkVC1759R		ACTTCCAATAGCGCCAGC		
fkVC0184F	VSP-I	ACTCAACTGCGGCAGAGCT	1,690	59
fkVC0186R		TGGTTGGTCTACACGGCATCG		
fkVC0515F	VSP-II	AGAATGGAGTTCAGAGTGTCG	2,289	56
fkVC0517R		GATTCTGCGACCGCAACCAG		
attB primers				
VPI2attF	VPI-2	AGAGTGAAAGTCGCCAAAGC	521	57
VPI2attR		GGGTGCAATTTCGCATGTTGC		
VSPIatt2F	VSP-I	GCATGATCAGGCGTGGAATCG	835	57
VSPIatt2R		AGGCTACATCAAGGTCGATGAG		
VSPIIattF	VSP-II	TGGTGGGTTAGAGTGCCGTC	397	59
VSPIIattR		CGTCATCCAAGCCGCTCAG		

InvVC0516F and primer InvVC0490R will amplify a 1,922-bp PCR product if a CI of VSP-II is formed (Table 2). A second set of inverse primer pairs was designed for each region to confirm the presence of CIs by nested PCR (Table 2). These inverse primer pairs were designed for sequences internal to the expected PCR product of the first inverse primer pair (Fig. 2). The PCRs of the first inverse PCR assays were used as templates for the nested PCR assays.

Construction of mutants. Deletion mutants were constructed by the splice-by-overlap-extension-(SOE)-PCR method for each of the integrase genes carried in the VPI-2 (VC1758), VSP-I (VC0185), and VSP-II (VC0516) regions, designated int_{VPI-2}, int_{VSP-I}, and int_{VSP-II}, respectively (12). SOE-PCR primers were designed to produce nonfunctioning constructs of the 1,235-bp int_{VPI-2}, the 1,214-bp int_{VSP-I}, and the 1,241-bp int_{VSP-II} genes. The sizes of the regions removed from the int_{VPI-2}, int_{VSP-I}, and int_{VSP-II} genes were 262 bp, 283 bp, and 293 bp, respectively, and were constructed with *V. cholerae* strain N16961 to create mutant strains *V. cholerae* RAM-1, RAM-2, and RAM-3, respectively (Table 1). All three mutant strains were constructed as follows. Primer pairs SOEVC1758a/SOEVC1758b and SOEVC1758c/SOEVC1758d were used to amplify PCR products of 387 bp and 422 bp, respectively, from VC1758 from the *V. cholerae* strain N16961 (Table 2). The resulting PCR products were purified and ligated together using T4 DNA ligase (New England Biolabs). The ligated product was amplified with the primer pair SOEVC1758a and SOEVC1758d, resulting in an 809-bp PCR product, which was restricted with enzymes XbaI and SacI, and ligated with pDS132 (New England Biolabs), resulting in pRM1. pRM1 was transformed into *E. coli* strain DH5αλpir, plasmid purified, and then transformed into *E. coli* β2155 cells. *E. coli* β2155 transformants were conjugated with *V. cholerae* N16961, and *V. cholerae* cells selected for pRM1 transfer were passaged to cure them of the integrated pRM1. Using 1 μl of these cultures as templates, PCR was used to screen for *V. cholerae* strains in which the wild-type genes were replaced by the mutant genes, which were confirmed by sequencing. The Δint_{VPI-2} strain was designated *V. cholerae* strain RAM-1. Similarly, knockout mutants were constructed with int_{VSP-I} (VC0185) and int_{VSP-II} (VC0516) in *V. cholerae* N16961 in the same manner by using the primer pairs listed in Table 2.

RESULTS

Genome structure and gene content of VPI-2, VSP-I, and VSP-II. Previously, we showed that the 57-kb VPI-2 region is present predominantly in *V. cholerae* O1 isolates and that among 13 of 14 O139 serogroup isolates examined, the region

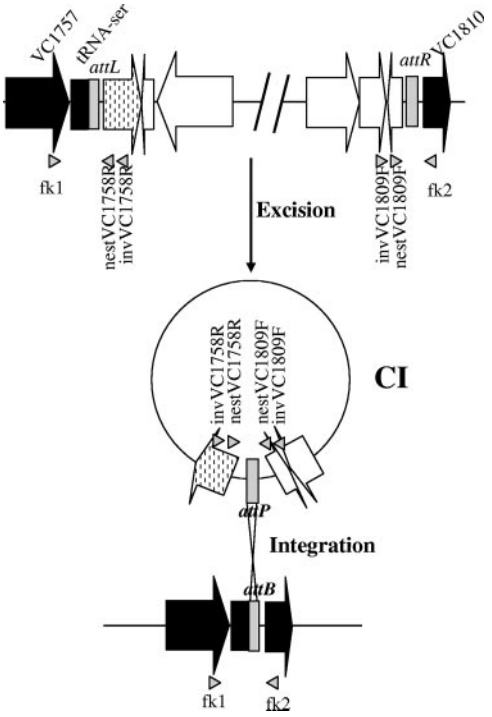

FIG. 2. Schematic representation of a partial VPI-2 region indicating the locations of primer pairs used to detect integrated, excised, circularized, and deleted VPI-2 regions. Black arrows represent core region genes. Boxes represent *att* sites. Triangles represent primers used in this study.

is truncated (Fig. 3) (14, 15). In these 13 O139 serogroup isolates, the VPI-2 region consisted of VC1758 (*int*), VC1759, a partial VC1760 sequence, and region VC1789 to VC1809, which encompasses the Mu phage-like region (Fig. 3). In the present analysis, we examined the 12 available sequenced genomes of *V. cholerae* and uncovered four additional variant VPI-2 regions at the tRNA-serine insertion site, all of which retained the sialic acid metabolism region (VC1773 to VC1784) and the P4-like integrase (VC1758) (Fig. 3). Of the *V. cholerae* O1 serogroup isolates examined, all showed VPI-2 structures and gene contents identical to the canonical VPI-2 region from strain N16961. Furthermore, in strain V52, an O37 serogroup clinical isolate recovered in 1968 from Sudan, the VPI-2 content was also identical to that of the *V. cholerae* O1 serogroup isolates. However, in *V. cholerae* strain MAK757, a pre-seventh pandemic El Tor strain recovered in the 1930s, and in strain B33, an El Tor isolate recovered in 2004 from Mozambique, the VPI-2 regions consisted of regions VC1758 to VC1790, which encodes the RM system and sialic acid metabolism, but the entire Mu phage-like region was absent as was ORFs VC1806 to VC1816, resulting in ORF VC1790 being adjacent to ORF VC1817 (Fig. 3). In *V. cholerae* strain NRT36S, a clinical O31 serogroup isolate from Africa, and strain AM-19226, an O39 serogroup strain from Bangladesh, the VPI-2 region contains ORFs VC1758, VC1773 to VC1784, and VC1789 to VC1809, and between ORFs VC1758 and VC1773 are genes that show homology to a type three secretion (TTS) system (Fig. 3) (3). In these strains, the RM system and the Mu phage regions are absent (Fig. 3). It is of interest to note that in two additional strains, strain 1587, an O12 serogroup isolate recovered from a patient in Peru, and strain 623-39, VPI-2 is similar in structure to that of AM-19226 and NRT36S; however, the TTS genes in these strains show on average only 90% homology to the TTS genes from strains AM-19226 and NRT36S. Strain 1587 also contains an additional deletion of ORFs VC1820 to VC1828. In *V. cholerae* strain V51, a clinical isolate recovered in 1987 in the United States, the VPI-2 region has a structure similar to that of strain AM-19226, except for the presence of an ~57-kb region directly downstream of the tRNA-ser locus between regions VC1757 and VC1758. This region shows extensive homology to phage genes (Fig. 3). Thus, the tRNA-ser locus is a hot spot for both PAI and phage insertion.

Comparative analysis of the VSP-I and VSP-II regions and their insertion sites among the sequenced *V. cholerae* genomes of O1 serogroup strains showed that when these regions are present, they are identical to those from strain N16961. However, in *V. cholerae* strain MZO-3, an O37 serogroup isolate from Bangladesh, at the VSP-I insertion site, a novel region is present, which we name *V. cholerae* island-4 (VCI-6) and is composed of 15 ORFs, including a transposase, an Ndp nucleoid-associated protein, HipA, a GTP cyclohydrolase, and an XerDC-like integrase (data not shown). In *V. cholerae* strain 623-39, at the VSP-II tRNA-methionine insertion site, an ~21 kb region is present, which we named VCI-5. The region encodes a UmuDC DNA repair system, a HipA homologue, and a number of ORFs that show homology to the GEI VPaI-1 (ORFs VP0380 to VP0403) identified in *V. parahaemolyticus* RIMD2210633 at the same tRNA-met locus (data not shown).

Excision potential of VPI-2. The VPI-2 regions among the sequenced *V. cholerae* genomes were conserved at the 5′ and 3′ insertion sites in most isolates, which prompted us to examine whether VPI-2 has the potential for excision from the genome to form an extrachromosomal CI. VPI-2 encodes a P4-like integrase (VC1758), a member of the tyrosine recombinase family, and is adjacent to a tRNA-serine locus (VC1757.1) (Fig. 1). The integrated VPI-2 region contains two direct repeat sequences, one at its 5′ end (*attL*) and one at its 3′ end (*attR*), which were detected by comparative sequence analysis of the VPI-2 positive and negative strains at the tRNA-serine site (Fig. 1). The VPI-2 *att* site appears in its complete 23-bp form at the 5′ end of the island (*attL*) associated with the tRNA-serine gene (Fig. 1). At this location, the first 15-bp segment of *attL* corresponds exactly to the last 15-bp segment of the tRNA-serine gene. This suggests that the tRNA-serine gene is the point where recombination occurred between the bacterial chromosome and the incoming CI of VPI-2. At the 3′ end of VPI-2, *attR* is 1 bp shorter than *attL* at 22 bp (Fig. 1). This is a common feature of the *att* sites and probably occurred during the recombination event that led to the integration of VPI-2.

Detection of circular excision products of VPI-2. The presence of the P4-like integrase and the putative *attL* and *attR* sites within VPI-2 suggests a possible mechanism of excision similar to that of the phage that encode the tyrosine recombinase family. To test this, the inverse PCR primer pair InvVC1758R and InvVC1809F was designed to amplify ORFs VC1758 and VC1809, respectively, such that a 2,053-bp PCR product will be obtained only if the VPI-2 region excises and forms a circular product (Table 2; Fig. 2). Inverse PCR was

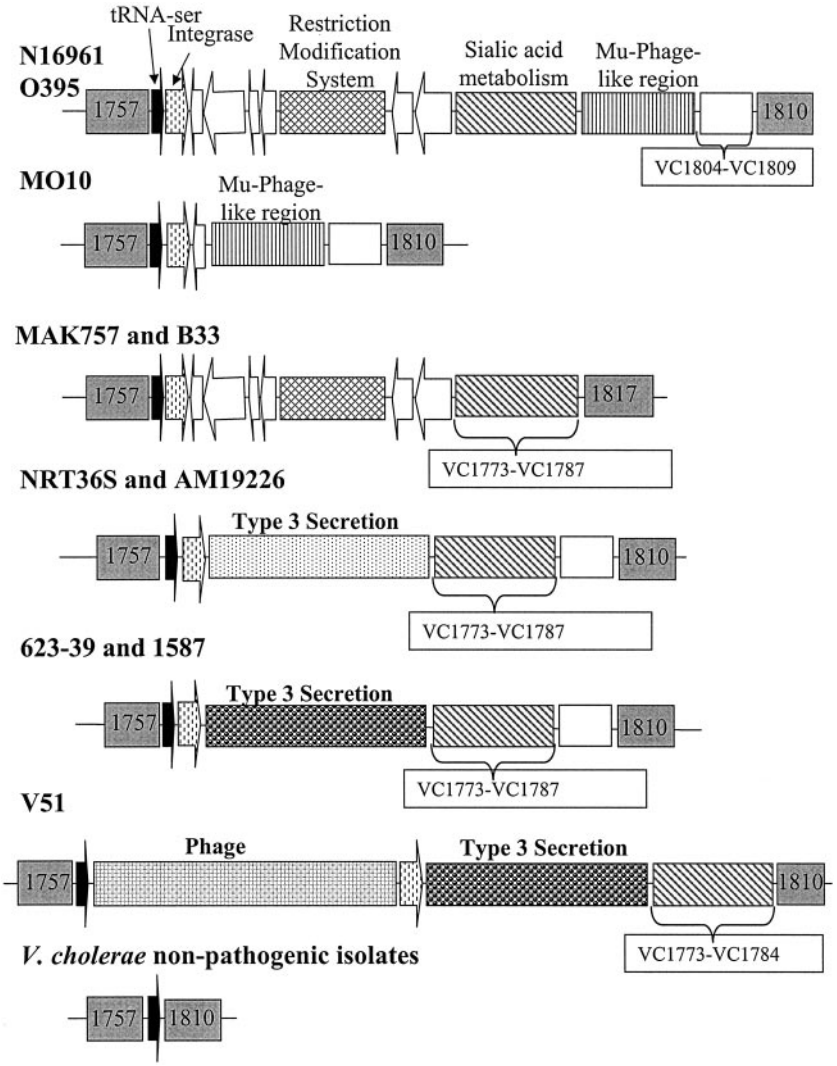

FIG. 3. Genetic structure and gene content of the canonical VPI-2 region in strains N16961 and O395 and the five variant VPI-2 regions from a range of sequenced genomes of *V. cholerae*. *V. cholerae* strain names are given along the left side. Gray boxes represent core chromosomal genes VC1757 and VC1810 or VC1817. Black arrows represent the tRNA-ser locus, and dashed arrows represent the integrase gene. Open arrows represent conserved hypothetical proteins, hatched boxes represent restriction modification regions, diagonally striped boxes represent sialic acid metabolism regions, vertical-lined boxes represent Mu phage regions, and dotted and bubble boxes represent type 3 secretion system regions.

performed with a plasmid DNA preparation from the *V. cholerae* O1 serogroup strains O395, N16961, E4, 2740-80, SG6, and O139, with MO2 and MO10 as templates. A range of PCR conditions were examined using this set of primers. In these inverse PCR assays, no detectable PCR product was obtained, which suggests that either VPI-2 does not excise from the chromosome or excision is not at a detectable level under the conditions we used to examined it (Fig. 4A). Therefore, we performed a second nested PCR assay using the first PCR as the template with the primer pair NestVC1809F/NestVC 1758R, designed to amplify an internal fragment of the inverse PCR product if it occurred at low levels (Table 2; Fig. 4B). An approximately 1.3-kb PCR product was amplified from six strains, O395, N16961, E4, 2740-80, MO2, and MO10, which indicates that VPI-2 did excise from its chromosomal insertion site and formed a circular excision product (Fig. 4B). The failure to show detectable PCR product in the initial PCR indicates that the excision of VPI-2 occurs but at very low levels under the conditions examined. No PCR product was obtained for strain SG6, a VPI-2-negative control strain. The resulting PCR products of the nested PCR assay were sequenced for all strains to confirm the excision event (see Fig. S1 in the supplemental material). From the sequenced PCR product, we identified the 23-bp *att* sequence of VPI-2, previously predicted from comparative sequence analysis, which was identical to that of *attL* found at the tRNA-serine locus.

Excision potential of VSP-I and VSP-II. As with VPI-2, the VSP-II attachment site was identified by comparative analysis between VSP-II positive and negative strains at the tRNA-methionine integration site. The 16-bp *att* sequence of VSP-II occurred in its complete form at the tRNA-methionine site (Fig. 1). The entire 16-bp sequence is also the first 16 bp of the DNA sequence of tRNA-methionine, suggesting that as with

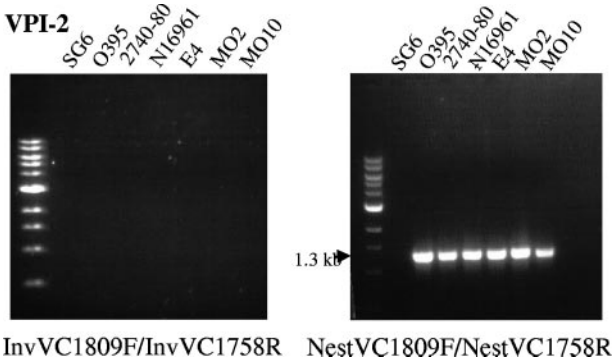

FIG. 4. PCR assay to detect CI of VPI-2 in *V. cholerae* strains SG6 (VPI-2-negative strain), O395, 2740–80, N16961, E4, MO2, and MO10 using the primer pair invVC1809F/invVC1758R and primer pair NestVC1809F/NestVC1758R. Lanes: 1, marker; 2; *V. cholerae* strain SG6 (VPI-2-negative strain); 3 to 6, *V. cholerae* O1 serogroup strains O395, 2740–80, N16961, and E4 (VPI-2 positive); 7 to 8, *V. cholerae* O139 strains MO2 and MO10 (VPI-2 positive).

VPI-2, the recombination/insertion of VSP-II occurred at this tRNA locus. At the 5′ end of VSP-II, the *attL* sequence is 2 bp shorter than the *attR* sequence. We could not identify an *att* sequence for VSP-I.

Detection of circular excision products of VSP-I and VSP-II. PCRs were performed using the inverse primers designed to detect the circularized forms of VSP-I and VSP-II (Table 2). Primers InvVC0185F/InvVC0175R were designed to amplify a 1,889-bp PCR product on excision of VSP-I, and primers InvVC0516F/InvVC490R were designed to give a 1,922-bp PCR product when VSP-II excises and circularizes (Table 2). For VSP-I, inverse PCR with primers InvVC0185F/InvVC 0175R amplified a product of approximately 1.9 kb for strains N16961, E4, MO2, and MO10 (strain O395 did not yield a product as it does not harbor VSP-I) (Fig. 5A). To confirm that the PCR product was generated from a VSP-I CI, a second nested PCR assay with primers NestVC0185F/NestVC0175R was performed using the first PCR as the template. As expected, PCR products of approximately 0.7 kb were amplified for *V. cholerae* strains N16961, E4, MO2, and MO10, confirming VSP-I excision and formation of a CI; no PCR product was obtained for strain O395, our negative control strain (Fig. 5A).

For VSP-II, inverse PCR with primers InvVC0516F/InvVC490R amplified PCR products of approximately 1.9 kb from strains N16961, E4, MO2, and MO10 (strain O395 did not yield a PCR product as it does not harbor VSP-II) (Fig. 5B). To confirm that the VSP-II PCR product was the result of

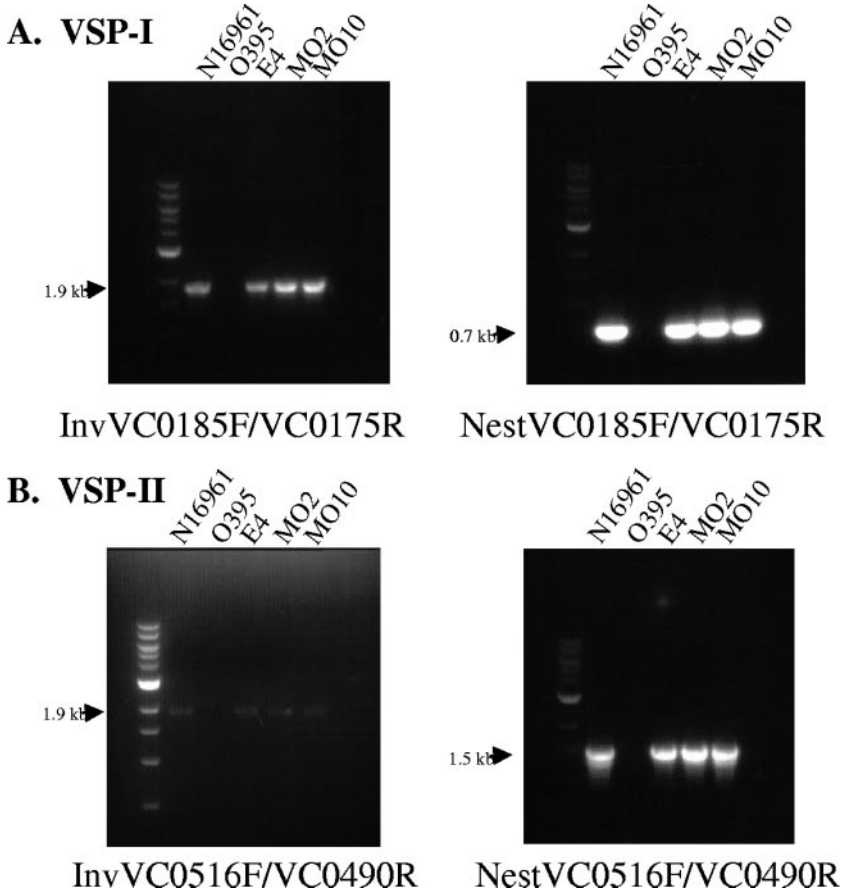

FIG. 5. PCR assays to detect CI of VSPI-I and VSP-II regions in *V. cholerae* strains N16961, O395, E4, MO2 and MO10. (A) VSP-I CI PCR assay using the primer pairs InvVC0185F/InvVC0175R and NestVC0185F/NestVC0175R. *V. cholerae* O395 is a VSP-I-negative control strain. (B) VSP-II CI PCR assay using primer pair InvVC0516F/InvVC0490R and NestVC0516F/NestVC0490R. *V. cholerae* O395 is a VSP-II-negative strain.

VSP-II excision and circularization, a second nested PCR was performed. Using the first inverse PCR product as the template with primers NestVC0516F/NestVC490R, approximately 1.5-kb PCR products were obtained for *V. cholerae* strains N16961, E4, MO2, and MO10. No PCR product was obtained for strain O395, our negative control (Fig. 5B). Sequencing was performed with the PCR products and gave the expected VSP-I and VSP-II excision sequences (see Fig. S2 and S3 in the supplemental material). Analysis of sequence data for VSP-I uncovered a potential *att* site consisting of 7 bp and also indicated that VSP-I is inserted at VC0186 (a gene encoding glutathione reductase). For VSP-II, the *attP* site was confirmed as a 16-bp sequence that was predicted by DNA sequence analysis. The *attP* gene found on the excised VSP-II is identical to that of *attR* and the first 16 bp of the gene tRNA-methionine site (Fig. S3).

Roles of VPI-2, VSP-I, and VSP-II integrase genes in excision. In order to investigate the roles of the int_{VPI-2}, int_{VSP-I}, and int_{VSP-II} genes in the excision of VPI-2, VSP-I, and VSP-II, respectively, deletion mutations were constructed in each of these genes in *V. cholerae* strain N16961. The resulting knockout mutant strains were designated *V. cholerae* RAM-1 (Δint_{VPI-2}), RAM-2 (Δint_{VSP-I}), and RAM-3 (Δint_{VSP-II}). Plasmid DNAs were isolated from RAM-1, RAM-2, and RAM-3 and used as the templates for inverse PCRs.

First we examined *V. cholerae* strains RAM-1, RAM-2, and RAM-3 for their abilities to excise VPI-2 (Fig. 6A). Strain RAM-1, which lacks the VPI-2 cognate integrase (int_{VPI-2}) did not amplify a CI product for VPI-2 in both the inverse and the nested PCR assays (Fig. 6A). Conversely, strains RAM-2, RAM-3, and N16961 amplified an ~1.3-kb PCR product with the nested primer pair for VPI-2 (Fig. 6A). All PCRs were repeated at least three times. These results show that VPI-2 cannot excise from the *V. cholerae* chromosome in the absence of a functional int_{VPI-2} gene; however, VPI-2 excision is not affected by deletions in int_{VSP-I} and int_{VSP-II}, since PCRs carried out with strains RAM-2 and RAM-3 gave PCR products identical to that of the wild type (Fig. 6A).

The int_{VSP-I} mutant strain RAM-2 was examined for VSP-I excision by inverse PCR with primers InvVC0185F/InvVC0175R, and no PCR product was obtained with RAM-2 as the template. Similarly, the second nested PCR failed to amplify a PCR product for the strain RAM-2 (Fig. 6B). These results indicate that a functional copy of int_{VSP-I} is required for excision. For VSP-I, with strains RAM-1 and RAM-3 as templates, PCR products were obtained for both inverse and nested PCRs that were similar to that of the wild type, indicating that these integrases are not required for VSP-I excision (Fig. 6B).

Inverse PCR was performed using the primer pair InvVC 0516F/InvVC490R, with *V. cholerae* strain RAM-3 as the template, which is the Δint_{VSP-II} mutant strain. As observed for both the Δint_{VPI-2} and the Δint_{VSP-I} mutants, no PCR product was obtained (Fig. 6C). A nested PCR with primers NestVC0 516F/NestVC0490R was employed and also failed to amplify the expected 1,493-bp product, whereas positive control strains gave the expected PCR product band (Fig. 6C). With both the RAM-1 and RAM-2 strains as templates, PCR products of the expected sizes were obtained (Fig. 6C). These results show that in *V. cholerae* strain RAM-3, int_{VSP-II} is required for excision. In summary, these results suggest that the cognate integrase gene for each island region is required for excision.

Effect of RecA on excision of VPI-2, VSP-I, and VSP-II. Plasmid DNA was isolated from *V. cholerae* strain Bah-3, a derivative of strain E7946 in which the *recA* recombinase gene, among others, has been knocked out (33). Strain Bah-3 was examined for the excision of VPI-2, VSP-I, and VSP-II, as described before, to determine if RecA plays any role in the excision of the three PAIs. For VPI-2, a two-stage nested PCR with primers NestVC1809F/NestVC1758R revealed a PCR product of 1.3 kb, identical to that amplified as shown in Fig. 6A, lane 7. Similarly, for both inverse and nested PCR assays, a PCR product of the expected size was obtained from both the VSP-I and the VSP-II regions, using Bah-3 as the template (Fig. 6B and C, lane 7). These results indicate that the excision of the three PAIs occurs in *V. cholerae* strain Bah-3 and that the RecA recombinase is not essential for excision, although it could have more subtle effects on the levels of PAI excision that could be undetectable by the techniques used in this study.

Detection of *attB* postexcision. The excision of a PAI and its subsequent circularization should, in theory, leave an "empty" insertion site, comparable to those in non-PAI-harboring strains such as *V. cholerae* strain O395, which is VSP-I and VSP-II negative. Primers were designed to amplify the insertion site (*attB*) of the three PAIs after the PAI had excised. To detect *attB* of VPI-2, the primer pair VPI2attF/VPI2attR was used (Table 2). In VPI-2-positive strains, this primer pair will be able to amplify a PCR product only if VPI-2 has excised. PCRs were carried out using 1 μl of overnight broth cultures of *V. cholerae* strains O395, 2740-80, N16961, E4, MO2, and MO10 as templates. For all the strains, the primer pair VPI2attF/VPI2attR amplified the expected ~0.5-kb product, which represents the insertion site of VPI-2 (the tRNA-serine site) postexcision. *V. cholerae* strain SG6 was employed as a negative control as it does not contain any novel region at the tRNA-serine locus. The PCR product was sequenced for the six VPI-2-positive strains and the one island-negative strain to confirm that the product was the VPI-2 insertion site and to determine the presence of the *attB* sequence of VPI-2 in the postexcision state and in the nonintegrated state. The sequence of the PCR product amplified from the VPI-2-positive strains was that of the "empty" VPI-2 insertion site, and the *attB* site postexcision was 22 bp long (see Fig. S4A in the supplemental material). The *attB* sequence was identical in all six VPI-2-positive strains tested. The *attB* sequence in the VPI-2-negative strain SG6 was identical to the 23-bp *attP* and *attL* sequences, which suggests that this strain did not contain the VPI-2 region and subsequently lose it.

As stated previously, we failed to identify possible *att* sites for VSP-I integration by sequence comparisons of VSP-I-positive and VSP-I-negative strains. We examined the nucleotide sequence of VSP-I from PCR products generated using the VSP-Iatt2F/VSP-Iatt2R primer pair in VSP-I-positive isolates to determine whether a possible *attB* site could be uncovered (see Fig. S4B in the supplemental material). We identified a 7-bp sequence that could be a possible integration site; however, this site is shorter than previously identified *att* sites, and in addition, the postexcision sequences *attL* and *attR* are all identical.

The primers VSPIIattF/VSPIIattR were designed to amplify

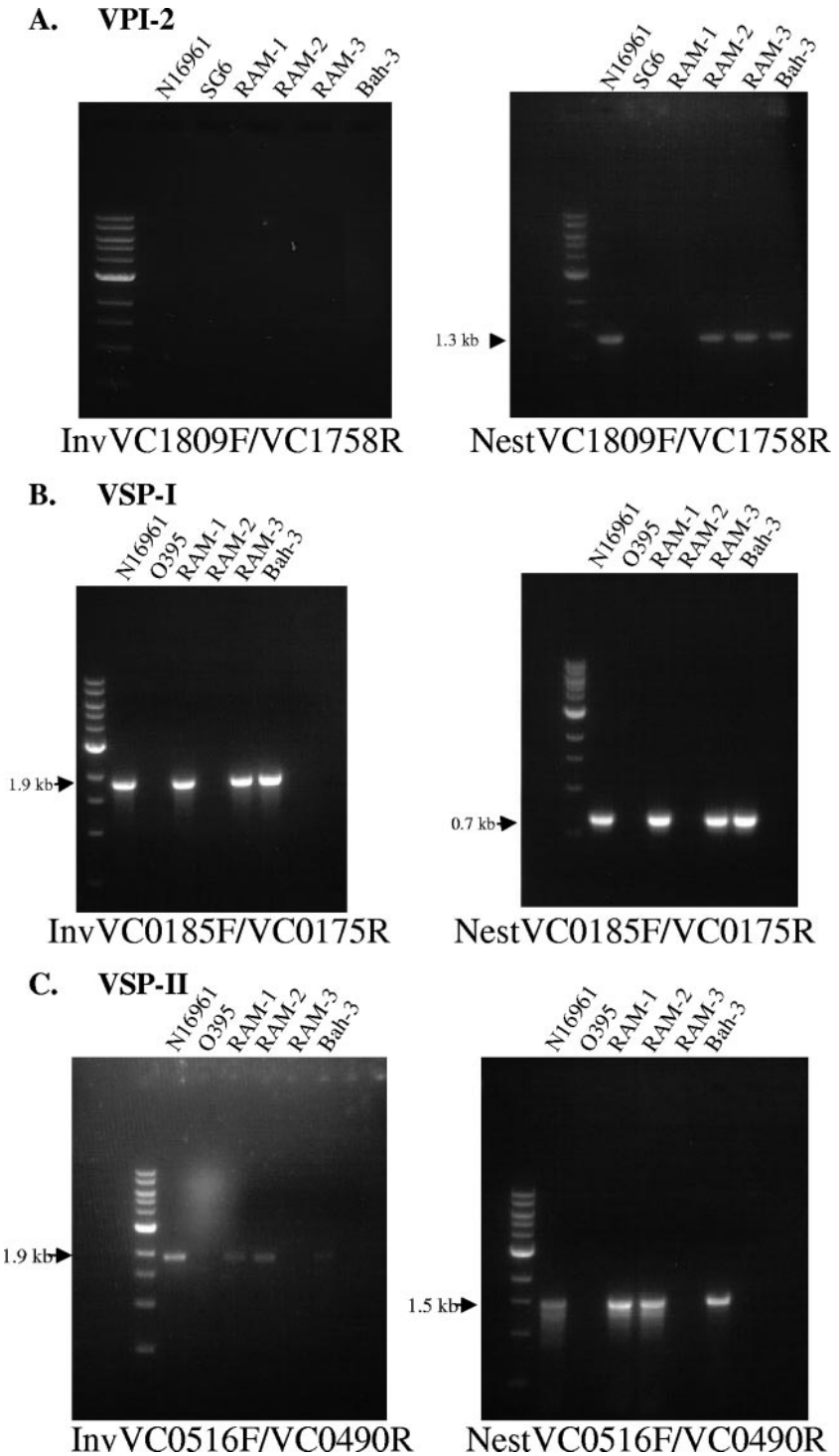

FIG. 6. PCR assays to detect CIs of VPI-2, VSP-I, and VSP-II in *V. cholerae* mutant strains RAM-1, RAM-2, and RAM-3. (A) Inverse and nested PCR assays of VPI-2 to detect excision product from strain RAM-1 ($\Delta int_{VPI\text{-}2}$). The expected PCR product was obtained for all strains except for RAM-1 ($\Delta int_{VPI\text{-}2}$) and SG6, the VPI-2-negative control. (B) Inverse and nested PCR assays of VSP-I to detect excision products for strain RAM-2. Inverse and nested PCR assays amplified a PCR product of the expected size from all strains except for RAM-2 ($\Delta int_{VSP\text{-}I}$) and O395, the negative control strain. (C) Inverse and nested PCR assays of VSP-II excision products for strain RAM-3 ($\Delta int_{VSP\text{-}I}$). PCR products were amplified from all strains except for RAM-3 and O395, the negative control strain.

the "empty" insertion site of VSP-II at the tRNA-met locus from VSP-II-positive and VSP-II-negative strains. Sequencing was performed with PCR products obtained to determine the *attB* site of VSP-II postexcision. Sequencing of the PCR product amplified from VSP-II-positive strains showed that, as with VPI-2, the postexcision *attB* sequence is the shorter version of the *att* sequence site, in this case, 14 bp, which is identical to that of the VSP-II *attL* sequence (see Fig. S4C in the supplemental material). The *attB* site of VSP-II was 16 bp in strains O395, V51, and V52, which are VSP-II-negative isolates, and was identical to the *attP* and *attR* sites of VSP-II, suggesting that these strains never contained the region.

DISCUSSSION

The acquisition of novel DNA via horizontal gene transfer and recombination has played a significant role in the emergence and reemergence of pathogenic bacteria. A number of studies have uncovered a range of mobile genetic elements among pathogenic *V. cholerae* isolates (4, 14, 16, 19, 22, 25, 35, 37). For example, four PAIs, VPI-1, VPI-2, VSP-I, and VSP-II, are associated with *V. cholerae* epidemic and pandemic isolates (4, 14, 16, 19, 25, 26). In this study, we reveal significant genetic heterogeneity within the VPI-2, VSP-I, and VSP-II PAIs among the sequenced genomes of *V. cholerae*. Within VPI-2, five possible variants of the canonical VPI-2 region from N16961 are described (Fig. 3). Among the O1 serogroup classical and El Tor biotype strains and V52, an O37 serogroup strain, the VPI-2 regions are identical. Strain V52 belongs to the *V. cholerae* O1 clonal complex and probably arose from an El Tor isolate that acquired a new O antigen, similar to the emergence of the O139 serogroup toxigenic isolates found in 1992 (1, 2, 26, 36, 38). Two of the variant VPI-2 structures have resulted from major deletion events. In *V. cholerae* strain MAK757, an El Tor isolate recovered in the 1930s, before the emergence of the seventh pandemic strains in 1961, most of the VPI-2 region is present, with the exception of ORFs VC1789 to VC1816, which include the Mu phage region (Fig. 3). A similar deletion is present in strain B33, an El Tor isolate recovered in a 2004 isolate from Mozambique. In most *V. cholerae* O139 serogroup strains, only ORFs VC1758 and VC1759 and VC1789 to VC1809 are present; the RM and sialic acid metabolism regions are absent (14, 15). In MO2, an O139 serogroup strain isolated in India in 1992, the entire VPI-2 region is present; however, O139 strains isolated after 1992 do not contain the region (Fig. 3) (14, 15). Mu phage is known to cause spontaneous deletion and insertion events in chromosomal DNA, and this may explain the truncation of VPI-2 in O139 isolates. We speculate that the reemergence of *V. cholerae* El Tor isolates as the predominant cause of cholera may be the result of reduced fitness of the O139 strains due to the loss of the VPI-2 region genes (14, 15). The sialometabolism gene cluster, including neuraminidase, was retained among all other pathogenic strains, and the loss of this region in O139 isolates may have resulted in reduced fitness in the human host. Sialic acid is an amino sugar present in the human intestine and a possible important source of carbon and nitrogen; therefore, carriage of VPI-2 by the O1 serogroup isolates could give them a competitive advantage. The ability to catabolize sialic acid is not prevalent among bacteria and is found mainly among enteric pathogens.

The Mu phage-like region and the RM system are also missing from the five *V. cholerae* nonO1/nonO139 serogroup strains V51, NRT36S, AM-19226, 1587, and 623-39. These strains contain ORFs VC1773 to VC1786, encoding the sialic acid metabolism homologues, and adjacent to the tRNA-serine locus in these five isolates is ORF VC1758 (integrase), followed by homologues of a TTS system (Fig. 3). Interestingly, the TTS genes in strain 1587, an O12 isolate from a patient in Peru, and strain 623-39 have only ~90% homology with the TTS genes in strains V51, AM19226, and NRT36S. Also, strain 1587 contains an additional deletion of ORFs VC1820 to VC1828, which suggests that isolates from South America may have a different origin. In *V. cholerae* strain V51, recovered in 1987 in the United States, the VPI-2 region has an ~57-kb prophage directly downstream of the tRNA-ser locus, between ORFs VC1757 and VC1758. Thus, this tRNA-ser locus is a hot spot for the insertion of a range of mobile genetic elements. It will be of interest to determine whether this phage is involved in the mobility and transfer of the VPI-2 region in this strain, since VPI-2 does not encode any known self mobility genes.

Among O1 serogroup El Tor and classical isolates, the VPI-2 region is highly conserved, suggesting that it arose only once in the O1 serogroup. The variant VPI-2 regions among the O1 and O139 isolates is likely due to Mu phage deletion events. Among non-O1/non-O139 isolates, VPI-2 is also conserved, all strains containing the TTS and sialic acid metabolism regions. Our speculative evolutionary scenario for the emergence of VPI-2 variants favors the view that the VPI-2 found in non-O1/non-O139 isolates is the ancestral structure. The TTS region was probably replaced in an O1 serogroup isolate by the RM system, and the addition of the Mu phage and this strain gave rise to the O1 clonal complex. The RM system may act as an addiction system in VPI-2, ensuring its maintenance in O1 isolates. Previously, we showed that in *V. mimicus*, a close relative of *V. cholerae*, at the tRNA-ser locus, neither the RM system nor the Mu phage region was present; however, ORFs VC1758 and VC1773 to VC1786 were found in a diverse collection of isolates examined. In *V. mimicus*, between ORFs VC1758 and VC1773 an unidentified region is present (15). Since strains of *V. mimicus* cause gastroenteritis by an unknown mechanism, we speculate that *V. mimicus* may also contain a TTS system and possible be the origin of the TTS system in *V. cholerae*.

We found that the VPI-2 regions from a range of *V. cholerae* O1 isolates can excise from the genome and form CIs. In addition, we demonstrate that the truncated VPI-2 region from *V. cholerae* O139 strain MO10 can excise and form CI molecules. Bioinformatic analysis of the additional variants of VPI-2 suggests that they too can excise and form CIs since they all contain the same P4-like tyrosine recombinases and intact *attL* and *attR* attachment sites. Within the *V. cholerae* N16961 genome sequence, there are five additional tRNA-serine genes, which are possible sites for VPI-2 insertion. However, only 17 bp of the 23-bp *att* site is conserved within these tRNA-serine genes, indicating that VPI-2 insertion is unlikely to occur.

When it was present, the VSP-I regions were identical among all *V. cholerae* O1 serogroup strains examined. In *V.*

cholerae strain MZO-3, an O37 isolate recovered in Bangladesh in 2001, at the VSP-I insertion site, an approximately 17-kb region is present, which consists of 15 ORFs, the last 4 of which show some homology to the last 4 ORFs of VSP-I, including the 3′ *int* gene. Interestingly, at the 5′ end, a transposase was present in strain MZO-3. VSP-I can excise and form a CI in all strains examined, and the VSP-I cognate integrase is required for excision.

O'Shea and colleagues found that VSP-II is a 27-kb region with some homology to the *V. vulnificus* island-1 (VVI-1), a region unique to *V. vulnificus* strain YJ016 (25). The VVI-1 region is inserted at the same genome location as that of VSP-II, at the tRNA-met site. At the tRNA-met locus in *V. cholerae* strain 623-39, a 21-kb region was present which showed homology to a *V. parahaemolyticus* island-1 region from *V. parahaemolyticus* strain RIMD2210633 (13, 29, 31). Similar to VPI-2, VSP-II can excise from the genome and form a CI, and we found that a functional copy of the *int* gene carried on VSP-II is required for excision. Within the *V. cholerae* N16961 genome there are nine tRNA-methionine loci; however, only a 10-bp region of the 16-bp *att* site is shared among these genes, indicating that it is highly improbable that VSP-II, after it excises, can integrate at any of these sites.

PAIs in several bacterial species have shown the ability to excise from their chromosomal insertion sites. These PAIs include the large PAI SPI7 of *Salmonella enterica*, the high-pathogenicity island of *Yersinia pseudotuberculosis*, several of the PAIs of uropathogenic *Escherichia coli*, and the VPI-1 region from *V. cholerae* (7, 11, 20, 21, 30). Common features of excision and circularization are apparent, with the presence of mobility genes (integrases and transposases) and the *att* sites of each PAI being of particular importance. For the most part, the fate of the circular excised islands is unknown. It has been shown in *V. cholerae* and *Y. pseudotuberculosis* that postexcision, the islands have the capability of reinserting into the genome at new locations. The VPI-1 was found inserted into chromosome 2 of *V. cholerae*, while the high-pathogenicity island of *Y. pseudotuberculosis* can insert into any of three tRNA-asn genes (20, 21, 30). A recent study examining the excision of the large *Pseudomonas aeruginosa* PAI PAPI-1 of strain PA14 showed that in a subpopulation of cells, PAPI-1 could exist as a CI after precise excision from its tRNA-lys integration site (28). PAPI-1 is related to integrative conjugative elements and encodes self-mobility genes and genes related to DNA replication. Qui and colleagues also identified an orthologue of the *soj* chromosome partitioning gene located on PAPI-1, mutations which lead to the deletion of PAPI-1 from strain PA14, and this gene also had a role in the excision and/or transfer of PAPI-1 (28). They proposed that *soj* may perform a novel function in PAPI-1 cells such as protecting PAPI-1 directly from degradation or indirectly by promoting integration (28). We identified possible *soj* homologues in VPI-2 and VSP-II, and these two genes were located at opposite ends of the island to their respective *int* genes, similar to that of the *soj* gene in PAPI-1. We also identified homologues of Vis, a recombination directional factor, in both VPI-2 (VC1785 and VC1809) and VSP-II (VC0497), proteins related to AlpA from *E. coli* that activate transcription of prophage CP4-57 integrase, stimulating excision (34). With the exception of strains MAK757, B33, and V51, ORFs VC1804 to VC1809 were highly conserved among the isolates examined, suggesting that these genes may have important functional roles. The transfer of PAIs among *V. cholerae* isolates is poorly understood. It is known that VPI-1 can be transferred between *V. cholerae* O1 serogroup strains via a transducing phage, CP-T1 (24). The excision and formation of CIs are probably the first steps in HGT of these elements, whether by conjugation, transduction, or transformation. The recent discovery that *V. cholerae* becomes naturally competent for the uptake of naked DNA in the presence of chitin, an abundant molecule in the aquatic environment, suggests a possible mechanism of HGT of PAIs among *V. cholerae* isolates (23).

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